JOURNAL OF VIROLOGY, Apr. 2011, p. 3833–3841 Vol. 85, No. 8
0022-538X/11/$12.00 doi:10.1128/JVI.02361-10

Interaction of Kaposi's Sarcoma-Associated Herpesvirus ORF59 with oriLyt Is Dependent on Binding with K-Rta[▽]

Cyprian C. Rossetto, Ni Ketut Susilarini, and Gregory S. Pari*

University of Nevada—Reno, School of Medicine, Department of Microbiology and Immunology, Reno Nevada 89557

Received 11 November 2010/Accepted 27 January 2011

Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8 (HHV-8) displays two distinct life stages, latency and lytic reactivation. Progression through the lytic cycle and replication of the viral genome constitute an essential step toward the production of infectious virus and human disease. KSHV K-RTA has been shown to be the major transactivator required for the initiation of lytic reactivation. In the transient-cotransfection replication assay, K-Rta is the only noncore protein required for DNA synthesis. K-Rta was shown to interact with both C/EBPα binding motifs and the R response elements (RRE) within oriLyt. It is postulated that K-Rta acts in part to facilitate the recruitment of replication factors to oriLyt. In order to define the role of K-Rta in the initiation of lytic DNA synthesis, we show an interaction with ORF59, the DNA polymerase processivity factor (PF), one of the eight virally encoded proteins necessary for origin-dependent DNA replication. Using the chromatin immunoprecipitation (ChIP) assay, both K-Rta and ORF59 interact with the RRE and C/EBPα binding motifs within oriLyt in cells harboring the KSHV bacterial artificial chromosome (BAC). A transient-transfection ChIP assay demonstrated that the interaction of ORF59 with oriLyt is dependent on binding with K-Rta and that ORF59 fails to bind to oriLyt in the absence of K-Rta. Also, using the cotransfection replication assay, overexpression of the interaction domain of K-Rta with ORF59 has a dominant negative effect on oriLyt amplification, suggesting that the interaction of K-Rta with ORF59 is essential for DNA synthesis and supporting the hypothesis that K-Rta facilitates the formation of a replication complex at oriLyt.

Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8 (HHV-8) is a gammaherpesvirus with a double-stranded DNA genome of 165 kb carrying over 80 genes. KSHV is the causative agent of Kaposi's sarcoma, primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD). A hallmark of all herpesvirus is their ability to establish lifelong latent infections and their subsequent reactivation to produce viral progeny. Latent replication and lytic replication are characterized by distinct gene expression profiles. Progression through the lytic cycle and replication of the viral genome are of unique importance because many studies suggest that lytic reactivation of KSHV is an essential pathogenic step in multiple human diseases.

We initially demonstrated that the products of eight virally encoded genes are required for KSHV origin-dependent DNA replication. They are ORF9 (a DNA polymerase), ORF6 (a single-stranded DNA [ssDNA] binding protein), ORF40/41 (a primase-associated factor), ORF44 (a helicase), ORF56 (a primase), ORF59 (a processivity factor), ORF50 (K-RTA) (a major transactivator), and K-bZIP (1). Although much emphasis was initially placed on K-bZIP as the origin binding initiator protein due to its homology to Epstein-Barr virus (EBV) Zta, our laboratory demonstrated that in the context of the virus genome, the deletion of K-bZIP leads to an enhanced growth phenotype (11). Subsequent studies showed that when K-Rta was overexpressed in the transient assay, K-bZIP was no longer required (22). Data suggest that K-bZIP interacts with the latency-associated nuclear protein (LANA) and acts to modulate the lytic and latent phases of the virus cycle but does not directly participate in lytic DNA replication (22).

Since it was demonstrated that K-bZIP plays a role in modulating lytic and latent DNA replication, logically the role of viral initiator protein of lytic DNA synthesis falls to K-Rta. The evidence for this hypothesis is based on the fact the K-Rta can interact with C/EBPα binding motifs in oriLyt, one of the critical *cis*-acting regions, in the absence of K-bZIP (22). K-Rta also interacts with the R response element (RRE) and activates a promoter consistent with the triggering of lytic DNA replication through the act of transcriptional activation. This is not unlike the mode of initiation of lytic DNA synthesis in human cytomegalovirus (HCMV). In HCMV, UL84 along with IE2 serves to activate a promoter within oriLyt, and UL84 also interacts with regions of oriLyt (C/EBPα binding motifs) and appears to recruit replication factors (unpublished data). Specifically, our laboratory and others demonstrated that UL44, the processivity factor, binds to UL84 (8, 10). Hence, the dual role of initiator proteins as transcriptional activators and recruitment factors is observed in other herpesviruses. In the case of Epstein-Barr virus, Zta is a transcriptional activator, binding to a promoter within EBV oriLyt, as well as functioning as a factor that interacts with members of the replication complex (helicase) (12). Therefore, it is logical to assume that the initiator protein for KSHV lytic DNA replication would interact with a component of the viral replication machinery. It is also assumed that the replication protein interacting with K-Rta (the putative initiator protein) would be

* Corresponding author. Mailing address: University of Nevada—Reno, Department of Microbiology, Howard Bldg. 210, Reno, NV 89557. Phone: (775) 784-4824. Fax: (775) 327-2332. E-mail: gpari@medicine.nevada.edu.

▽ Published ahead of print on 2 February 2011.

a factor that is known to interact with DNA and may have some intrinsic enzymatic function that would facilitate DNA replication and at the same time recruit other replication factors to the site of initiation. ORF59 has all these characteristics, and as mentioned above, homologs of this protein were shown to be involved in the initiation of lytic DNA synthesis in other herpesvirus systems.

In this report we show that K-Rta interacts with ORF59. We demonstrate that ORF59 interacts with the C/EBPα binding motifs within oriLyt and that this binding is dependent upon the presence of K-Rta. These results suggest that K-Rta acts as the initiator protein by recruiting ORF59 to oriLyt. Lastly, using the cotransfection replication assay, we show that dominant negative inhibition of the K-Rta–ORF59 interaction abrogates oriLyt-dependent DNA replication. This strongly suggests that the K-Rta–ORF59 interaction is required for lytic DNA replication.

MATERIALS AND METHODS

Vero and HEK293 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% bovine growth serum (HyClone, Logan, UT). BAC36, the wild-type HHV-8 BACmid, was provided by S. Gao (University of Texas). Vero cells containing the bacterial artificial chromosome (BAC) constructs were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine growth serum and 250 μg/ml hygromycin. MECK cells containing BAC36, provided by E. Mesri (University of Miami), were maintained in DMEM plus 30% fetal bovine serum (FBS) (Atlanta Biologicals), 0.2 mg/ml endothelial growth factor (EGF) (Sigma, St. Louis, MO), 0.2 mg/ml endothelial cell growth factor supplement (ECGS) (Sigma, St. Louis, MO), 1.2 mg/ml heparin (Sigma, St. Louis, MO), insulin transferrin selenium (Invitrogen, Carlsbad, CA), and BME vitamin (VWR Scientific, Rochester, NY).

Coimmunoprecipitation. For cotransfection, HEK293 cells (2×10^6/10-cm dish) were transfected with ORF50 and ORF59 plasmids using TransIT LT1 (Mirrus). At 48 h posttransfection, protein extracts were prepared using lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% NP-40, and protease inhibitors), passed through a 22-gauge needle to shear the DNA, and centrifuged at 10,000 × *g* for 10 min to remove debris. The lysate was precleared with mouse IgG-agarose conjugate (Santa Cruz Biotechnology) at 4°C for 30 min, and then 50 μl of antihemagglutinin (anti-HA) or anti-FLAG affinity agarose gel (Sigma) was added to the lysate. This mixture was rotated 4°C overnight. The beads were then washed four times with 1 ml of Tris-buffered saline (Tris-HCl [pH 7.4], 150 mM NaCl), each time with rotation for 10 min at 4°C. After the final wash, the beads were resuspended in 125 μl Laemmli sample buffer (Bio-Rad) and boiled for 5 min. Twenty microliters of the immunoprecipitated protein was separated through a 10% SDS-polyacrylamide gel, which was subsequently transferred to an Immun-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After an initial blocking step (15 min with 0.1% Tween 20 in Tris-buffered saline plus 5% nonfat milk), the blots were reacted with anti-HA, anti-FLAG, anti-ORF59, or anti-K-Rta antibodies overnight at 4°C, followed by washing (15 min with 0.1% Tween 20 in Tris-buffered saline) and incubation for 30 min with horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using a chemiluminescence substrate (Femto; Pierce).

ChIP. For induced samples, a 293L cell line (4×10^7 cells) containing wild-type BAC36 was treated with phorbol 12-myristate 13-acetate (TPA) (25 ng/ml) for 5 days. For uninduced cells, cultures were grown to the same density and both samples were treated the same. For the transfected chromatin immunoprecipitation (ChIP) assay, 0.5 μg of oriLyt plasmid (8088sc) along with 5.0 μg of ORF59-FLAG and/or 5.0 μg of ORF50-FLAG or ORF50-HA was transfected into Vero cells and harvested at 72 h posttransfection. The protocol for the ChIP is modified from the Active Motif ChIP-IT express kit (catalog no. 53008). Cells were washed once with phosphate-buffered saline (PBS), fixed in a 1% formaldehyde–PBS solution for 10 min, and then washed twice in PBS. Three milliliters of PBS was added to the cells, and the cells were scraped, spun down at 1,500 × *g*, resuspended in 750 μl of lysis buffer plus protease inhibitors, and incubated on ice for 30 min. This solution was then sonicated 10 times for 10 s each pulse. Sonicated samples were analyzed by agarose gel electrophoresis prior to further use. Prior to immunoprecipitation, 10 μl was removed for the "input" sample. Sheared samples (161 μl) were incubated for 15 h at 4°C with 25 μl of magnetic beads, 10 μl ChIP buffer 1, 3 μl antibody (anti-FLAG, anti-ORF59, or isotype control monoclonal antibody 84), and 1 μl protease inhibitor cocktail (Sigma). Where noted, anti-FLAG M2 magnetic beads (Sigma) were used in place of the magnetic beads plus antibody. After incubation, the magnetic beads were collected by being placed on a magnetic stand and the supernatant was removed. Beads were washed once with 800 μl ChIP buffer 1, followed by two washes with 800 μl ChIP buffer 2, with rotation for 10 min at 4°C after the addition of each wash buffer. Beads were then resuspended with 50 μl of elution buffer AM2 and incubated for 15 min room temperature. Fifty microliters of reverse cross-link buffer was added, beads were quickly pelleted, and supernatant was transferred to a new tube. Eighty-eight microliters of ChIP buffer 2 and 2 μl of 5 M NaCl were added to "input" samples. All samples and input were then incubated at 94°C for 15 min. Two microliters of proteinase K solution was added and incubated at 37°C for 1 h, and finally 2 μl of proteinase K stop solution was added. This mixture was used for PCR analysis. Primers used to amplify C/EBPα sites (nucleotides [nt] 23326 to 23572) were forward primer 5′-AATCCCCCAT AATCCTCTGC-3′ and reverse primer 5′-GGAAAAATCAAAACAAAACTC-3′. Primers used to amplify the RRE within oriLyt (nt 24038 to 24287) were forward primer 5′-CTCTGGGTGGTTTCGGTAGA-3′ and reverse primer 5′-CCTCGTTACGGGTAAATCCA-3′. Control primers used to amplify an unrelated region within ORF45 were forward primer 5′-ACGTCCGGAGAGTTGG AACTGTCA-3′ and reverse primer 5′-GGGGTCCATGGGATGGGTTAGTC A-3′. Control primers used to amplify the ORF45 loci were forward primer 5′-ACGTCCGGAGAGTTGGAACTGTCAT-3′ and reverse primer 5′-GGCG TCCATGGGATGGGTTAGTCAG-3′.

Cotransfection replication assay. Vero cells were transfected with plasmids encoding HHV-8 oriLyt as well as the complete set of core replication proteins, ORF6, ORF9, ORF40/41, ORF44, ORF56, ORF59, K-bZIP, K-Rta, and K-Rta 350-550aa-HA (containing the region of K-Rta from amino acid [aa] 350 to 550). Cells were harvested 5 to 7 days posttransfection, and total cellular DNA was isolated and cleaved with EcoRI and DpnI. Cleaved DNA was separated using a 0.8% agarose gel, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled-pGEM probe.

RESULTS

K-Rta interacts with ORF59. Both the transient-cotransfection replication assay and BACmid recombinants showed that K-bZIP is not directly required for lytic DNA synthesis but may play a role in modulation or facilitation of initiation of the lytic cycle (11, 22). Hence, the only noncore protein absolutely required for oriLyt-dependent DNA replication is K-Rta encoded by ORF50. Logically it must be assumed that K-Rta is the major factor involved in initiation and possible recruitment of the replication complex to oriLyt. Several other lines of evidence point to K-Rta as the initiator protein for lytic DNA replication: (i) K-Rta was shown to interact with the C/EBPα binding motifs within oriLyt and the RRE, (ii) K-Rta can interact with the C/EBPα in the absence of K-bZIP, and (iii) K-Rta appears to have both a transactivation function and a yet-undefined role in DNA replication and is a component of replication compartments (27). In an effort to elucidate the mechanism(s) involved in the initiation of lytic DNA replication mediated by K-Rta, we investigated the interaction of K-Rta with members of the replication complex. The reason for undertaking this analysis was that it is logical to assume that an initiator protein would interact with at least one of the proteins required for the enzymatic synthesis of DNA. This is the case for all other studied herpesvirus initiator proteins (HCMV UL84 binds to UL44, herpes simplex virus type 1 [HSV-1] UL9 binds to UL42 and ssDNA binding protein, and EBV Zta binds to helicase). We evaluated the ability of K-Rta to interact with any of the identified core replication proteins by cotransfection and immunoprecipitation using differentially tagged expressed proteins (not shown). As a result of these

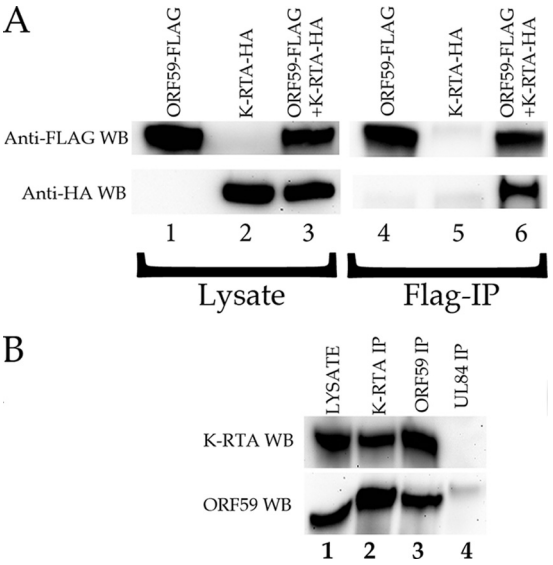

FIG. 1. ORF59 interacts with K-Rta. (A) HEK293 cells were cotransfected with plasmids expressing FLAG-tagged K-Rta and HA-tagged K-Rta. Protein lysates were prepared, and protein-protein interactions were detected using anti-FLAG antibody and reacting Western blots (WB) with anti-HA antibody. Lanes: 1 and 4, transfection of ORF59-FLAG expression plasmid; 2 and 5, transfection of K-Rta-HA; 3 and 6, cotransfection of ORF59-FLAG and K-Rta-HA. Antibodies used for Western blotting are show on the left. (B) ORF59 interacts with K-Rta in KSHV-infected cells. Coimmunoprecipitations were performed using BCBL-1 protein extracts from cells treated for 4 days with TPA/*n*-butyrate. Lanes: 1, cell lysates; 2, immunoprecipitation using anti-K-Rta-specific antibody; 3, immunoprecipitation using anti-ORF59-specific antibody; 4, immunoprecipitation using anti-UL84-specific antibody.

experiments, we found that K-Rta interacted with ORF59 (a polymerase processivity factor), which was also shown to interact with oriLyt as demonstrated by a recent proteomics assay (26). Since ORF59 is an essential component of the replication complex, it was logical to evaluate the interaction of this protein with K-Rta, a protein that interacts with oriLyt a two specific locations within oriLyt. Also, the proteomics assay was performed by using oriLyt as bait and revealed two regions of oriLyt where ORF59 was shown to bind. Those regions correlated with K-Rta binding sites identified earlier, the C/EBPα binding motifs and the RRE (14, 27). To investigate a possible interaction between K-Rta and ORF59, we cotransfected HEK293 cells with an ORF59-HA-tagged expression plasmid along with a K-Rta-FLAG-tagged expression plasmid. Protein lysates were prepared at 2 days posttransfection, and protein complexes were immunoprecipitated using anti-FLAG antibodies. Immunoprecipitated protein was resolved using an SDS-polyacrylamide gel, and protein interactions were visualized by Western blotting using anti-HA antibodies. K-Rta was efficiently immunoprecipitated using anti-FLAG antibodies, indicating that the two proteins interact in transfected cells (Fig. 1A, lane 5). The control lane, using anti-FLAG for the immunoprecipitation for protein lysates generated from cells transfected with ORF50-HA, failed to show a specific protein band (Fig. 1A, lane 6). These data established an interaction between K-Rta and ORF59.

In order to confirm the interaction of ORF59 with K-Rta in the infected-cell environment, we repeated the coimmunoprecipitation using TPA/*n*-butyrate-induced BCBL-1 cells. Cell lysates were prepared at 4 days postinduction, and protein-protein complexes were immunoprecipitated using either ORF59- or K-Rta-specific antibodies. A control immunoprecipitation was performed using HCMV anti-UL84-specific antibody. Western blots show that ORF59 was efficiently immunoprecipitated using the anti-K-Rta antibody and that K-Rta was detected using anti-ORF59-specific antibody from BCBL-1 induced cells (Fig. 1B, lanes 2 and 3, respectively). The control immunoprecipitation failed to show any specific immunoprecipitated band (Fig. 1B, lane 4). Hence, this experiment strongly suggests that ORF59 interacts with K-Rta in KSHV-infected cells during the lytic phase.

Mapping of ORF59–K-Rta interaction domains. It was previously shown that ORF59 forms a self-interaction (dimer) (2, 3, 5). The dimerization domains were shown to be localized to two regions of the protein between aa 1 and 21 and aa 277 and 304 (4). In order to elucidate the domain for interaction of ORF59 with K-Rta, we cotransfected HEK cells with plasmid expression vectors expressing various FLAG-tagged fragments of ORF59 along with full-length HA-tagged K-Rta (Fig. 2A). Protein lysates were prepared, and protein complexes were immunoprecipitated using anti-FLAG antibody and analyzed by Western blotting with anti-K-Rta antibody to detect protein interactions. ORF59 fragments from amino acids 199 to 396 and 266 to 396 interacted with K-Rta (Fig. 2B, lanes 4 and 5); however, a protein fragment from amino acid 1 to 199 failed to pull down full-length K-Rta (Fig. 2B, lane 3). Control transfections, where either full-length ORF59 and K-Rta were shown to interact or the ORF59 was omitted, were also included (Fig. 2B, lanes 1 and 2). These experiments indicated that the interaction domain for ORF59 was between amino acids 266 to 396 of the ORF59 protein. This region of the ORF59 protein contains a previously identified polymerase interaction domain, the double-stranded DNA binding domain (Fig. 2A).

ORF59 interacts with the C/EBPα and RRE binding motifs within oriLyt. Since we determined that ORF59 and K-Rta interact, we next wanted to determine if ORF59 binds to the same DNA sequences reported to interact with K-Rta, namely, the RRE and the C/EBPα binding motifs. We employed the ChIP assay to investigate the potential for ORF59 to interact with the RRE and C/EBPα binding motifs using antibodies specific for ORF59 in cells harboring the KSVH BAC36 induced with TPA. BAC36-containing HEK293 cells were treated with TPA, and a ChIP was performed at 3 days posttreatment. In KSHV BAC36-infected cell lines, ORF59 was shown to interact with the regions of oriLyt DNA containing either C/EBPα binding motifs or the RRE binding motif within oriLyt (Fig. 3, left and middle panels, respectively, lanes anti-ORF59). The control immunoprecipitation, using the unrelated antibody specific for HCMV UL84 to detect a PCR product when using primers flanking the oriLyt RRE element or C/EBPα binding motifs, failed to yield an amplified product (Fig. 3, lanes anti-UL84). Also, a control PCR was performed using primers specific for the ORF45 region of the genome, which failed to produce a detectable amplified product (Fig. 3, right panel). These experiments demonstrated that ORF59 interacts with regions of the KSHV oriLyt that were previously

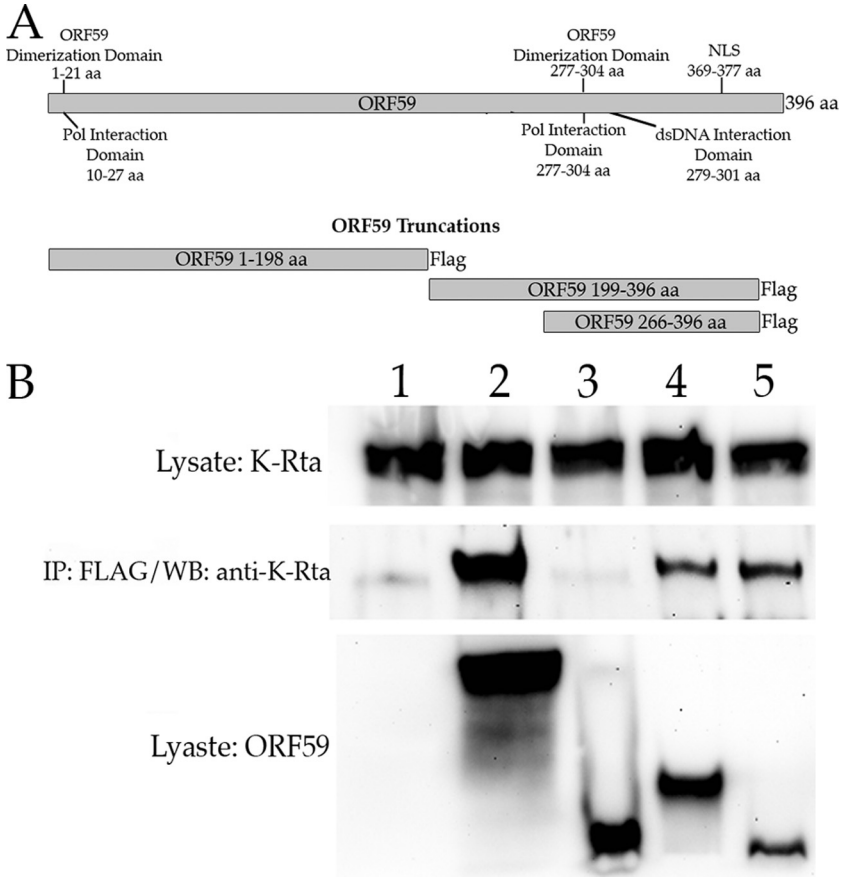

FIG. 2. Amino acids 266 to 396 of ORF59 interact with K-Rta. Coimmunoprecipitation of various truncations of the ORF59 protein with full-length K-Rta is shown. (A) Schematic of ORF59 full-length protein and fragments used in cotransfection assays. Also shown are various identified protein interaction domains. (B) HEK293 cells were cotransfected with a plasmid expressing HA-tagged K-Rta and plasmids expressing HA-tagged full-length ORF59 or peptide fragments of ORF59. Lanes: 1, transfection of K-Rta expression plasmid; 2, cotransfection of full-length ORF59 and K-Rta expression plasmids; 3, cotransfection of ORF59 aa 1 to 198 expression plasmid and the K-Rta expression plasmid; 4, cotransfection of ORF59 aa 199 to 396 expression plasmid and the K-Rta expression plasmid; 5, cotransfection of ORF59 aa 266 to 396 expression plasmid and the K-Rta expression plasmid.

shown to interact with K-Rta. These data strengthen the hypothesis that K-Rta is the initiator protein and ORF59 is involved in the lytic DNA synthesis initiation process.

ORF59 binding is dependent on the interaction of K-Rta with oriLyt. In order to characterize the binding of ORF59 to oriLyt, we examined the binding of ORF59 and K-Rta to oriLyt in the absence of any other viral protein. The rationale for this is to determine if ORF59 interacts with oriLyt as part of a protein complex with K-Rta. If this type of interaction (cooperative binding) occurs, then this would strengthen the argument that K-Rta recruits other replication proteins to oriLyt and hence would strongly suggest that K-Rta plays a major role in the initiation of lytic DNA synthesis by facilitating the recruitment of replication factors to oriLyt.

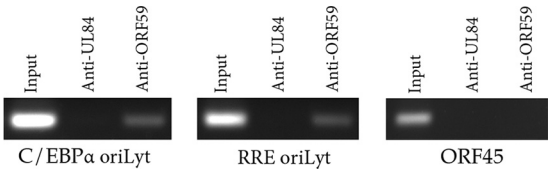

FIG. 3. ORF59 interacts with regions of oriLyt that contain C/EBPα binding motifs and the RRE. ChIP assay of the BACmid-infected 293L or MECK cell line is shown. Lanes show input DNA, control antibody (anti-UL84), and anti-ORF59 antibody. Primer sequences for amplification of RRE region: forward, 5′-CTCTGGGTG GTTTCGGTAGA-3′; reverse, 5′-CCTCGTTACGGGTAAATCCA-3′. Primer sequences for amplification of C/EBPα oriLyt region: forward, 5′-AATCCCCCATAATCCTCTGC-3′; reverse,5′-GGAAA AATCAAAACAAAACTC-3′.

In order to examine the interaction of K-Rta and ORF59 with oriLyt in a cellular environment, we needed to perform ChIP assays using a transfection protocol. This would in essence "isolate" these two viral factors with the goal of determining if the binding of one (ORF59) is dependent on the presence and binding of the other (K-Rta) to oriLyt. Vero cells were used to develop a modified ChIP assay by transfecting the oriLyt-L-containing plasmid along with plasmids expressing tagged ORF59 and/or K-Rta proteins. We used ChIP PCR primers that amplified the region of oriLyt (C/EBPα binding motifs) that was previously shown to interact with K-Rta and ORF59 (see above). Cells that had only the oriLyt plasmid transfected showed no PCR signal, as expected (Fig. 4, group

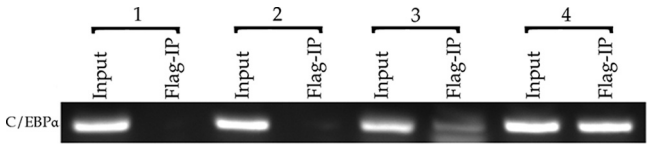

FIG. 4. The presence of K-Rta influences the interaction of ORF59 with oriLyt. Vero cells were transfected with plasmids expressing K-Rta and/or ORF59 along with the oriLyt-containing plasmid. ChIP assays were performed using anti-K-Rta or anti-ORF50 antibody and primers specific for the C/EBPα binding motifs within oriLyt. Group 1, ChIP assay from cells transfected with oriLyt-containing plasmid using anti-FLAG antibody. Group 2, ChIP assay from cells transfected with the ORF59-FLAG expression plasmid plus the oriLyt-containing plasmid using the anti-FLAG antibody. Group 3, ChIP assay from cells transfected with the K-Rta-FLAG expression plasmid plus the oriLyt-containing plasmid using the anti-FLAG antibody. Group 4, ChIP assay from cells transfected with the ORF59-FLAG and K-Rta-HA expression plasmids plus the oriLyt-containing plasmid using anti-FLAG antibody.

1). Interestingly, ORF59 was unable to interact (or interacted weakly) with oriLyt when expressed in cells transfected with the oriLyt-containing plasmid (group 2). As expected, K-Rta did interact with oriLyt in the absence of any other viral proteins (Fig. 4, group 3). However, ORF59 was able to associate with oriLyt DNA in the presence of K-Rta, as indicated by a positive PCR product when ORF59-FLAG-tagged protein was pulled down using an anti-FLAG antibody (Fig. 4, group 4). These data show that ORF59 can interact with oriLyt in a cooperative manner with K-Rta.

These experiments strongly suggest that ORF59 interacts with regions of oriLyt that are that same as those identified as a substrate for K-Rta. These data, coupled with the coimmunoprecipitation data demonstrating an interaction between ORF59 and K-Rta, implicate both proteins as key factors in the initiation of lytic DNA synthesis.

Identification of K-Rta interaction domain. Since we determined that the domain of ORF59 that interacts with K-Rta contains several other interaction motifs, including the dimerization domain, we next wanted to identify the binding domain of the K-Rta ORF that interacts with ORF59. To this end, we generated a series of deletion mutants such that 100 amino acids were deleted across the entire K-Rta ORF (Fig. 5). Each one of these K-Rta deletion mutant plasmids was used in a cotransfection along with an expression plasmid for full-length ORF59. Lysates for both ORF59-FLAG full-length protein and the HA-tagged K-Rta deletion mutant proteins indicated that adequate expression was achieved (Fig. 5B, rows WB: lysate anti-FLAG and WB: lysate anti-HA). All K-Rta deletion mutants except K-RtaΔ401-500aa interacted with ORF59, strongly suggesting that this was the region where K-Rta bound with ORF59 (Fig. 5B, lane 7).

ORF59 dimerization domains are not required for interaction with K-Rta. The dimerization domains for ORF59 were previously identified as amino acids 1 to 21 and 276 to 305. To determine if the interaction of ORF59 with K-Rta was dependent on dimerization or involved the dimerization domains, we generated an ORF59-FLAG-tagged mutant where both of these homodimerization domains were deleted. We used this expression plasmid in a coimmunoprecipitation assay with full-length K-Rta-HA. The ORF59 homodimerization mutant protein was capable of interacting with K-Rta, indicating that an ORF59 self-interaction was not required for binding with K-Rta (Fig. 6, lane 3).

Dominant negative inhibition of oriLyt amplification by overexpression of the K-Rta interaction domain with ORF59. Using the information gleaned from our deletion mutants, we subcloned the small fragment of K-Rta that contains the ORF59 binding domain into an expression vector and provided an in-frame HA tag. We generated this plasmid such that the expressed peptide fragment was slightly larger than the domain we identified in the K-Rta deletion mutant assay, covering amino acids 350 to 550 of K-Rta. We cotransfected K-Rta 350-550aa-HA along with full-length ORF59-FLAG and then proceeded with an anti-FLAG antibody immunoprecipitation. In Fig. 7A the FLAG Western blot shows a very light band for ORF59-FLAG in the lysate column, but after FLAG-IP the ORF59 band appears much stronger. FLAG agarose beads were run along with the lysate and FLAG-IP to help distinguish between the ORF59 band and the antibody heavy-chain band; both bands are noted in the figure. The expression of the K-Rta 350-550aa-HA fragment is shown in the HA Western blot lysate column. The FLAG-IP column demonstrates that K-Rta 350-550aa-HA could be pulled down with full-length ORF59-FLAG.

Since our hypothesis is that K-Rta is the oriLyt initiation factor and one function is to recruit other members of the replication complex to oriLyt, we wanted to investigate whether the interaction between ORF59 and K-Ra is essential for the replication function of either protein. The use of a functional assay to assess the role of a specific protein in DNA replication is paramount to elucidate essential interactions. For HCMV, we have used the cotransfection replication assay coupled with the overexpression of protein binding domains to show that specific protein-protein interactions are required for functional activity in lytic DNA replication (7). Overexpression of the specific interaction domain of K-Rta with ORF59 in the cotransfection replication assay should competitively inhibit the interaction of K-Rta with ORF59. We performed the cotransfection replication assay using all of the required replication proteins along with K-Rta and oriLyt. In order to observe the effect of overexpression of the interaction domain of K-Rta with ORF59 in the replication assay, we included a plasmid (pK-Rta 350-550aa) that expressed the fragment from aa 350 to 550 in the cotransfection assay. We observed a decrease in oriLyt amplification upon addition of 1 μg of pK-Rta 350-550aa and a complete suppression when 3 μg of plasmid was included in the cotransfection mixture compared to the control transfection (Fig. 7, lanes 1 to 3). The control sample, where the ORF59 expression plasmid was omitted from the transfection mixture, showed no amplification of oriLyt (Fig. 7, lane 4). These results strongly suggest that the region of K-Rta from amino acid 350 to 550 can function as a dominant negative inhibitor of oriLyt amplification. This also suggests that we are inhibiting the interaction of K-Rta with ORF59 and that this protein-protein interaction is essential for oriLyt-dependent DNA replication.

As a control, we also evaluated the ability of K-Rta 350-550aa to suppress the transactivation activity of K-Rta. This experiment was performed to ensure that the dominant negative effect was not due to an unrelated inhibition of K-Rta

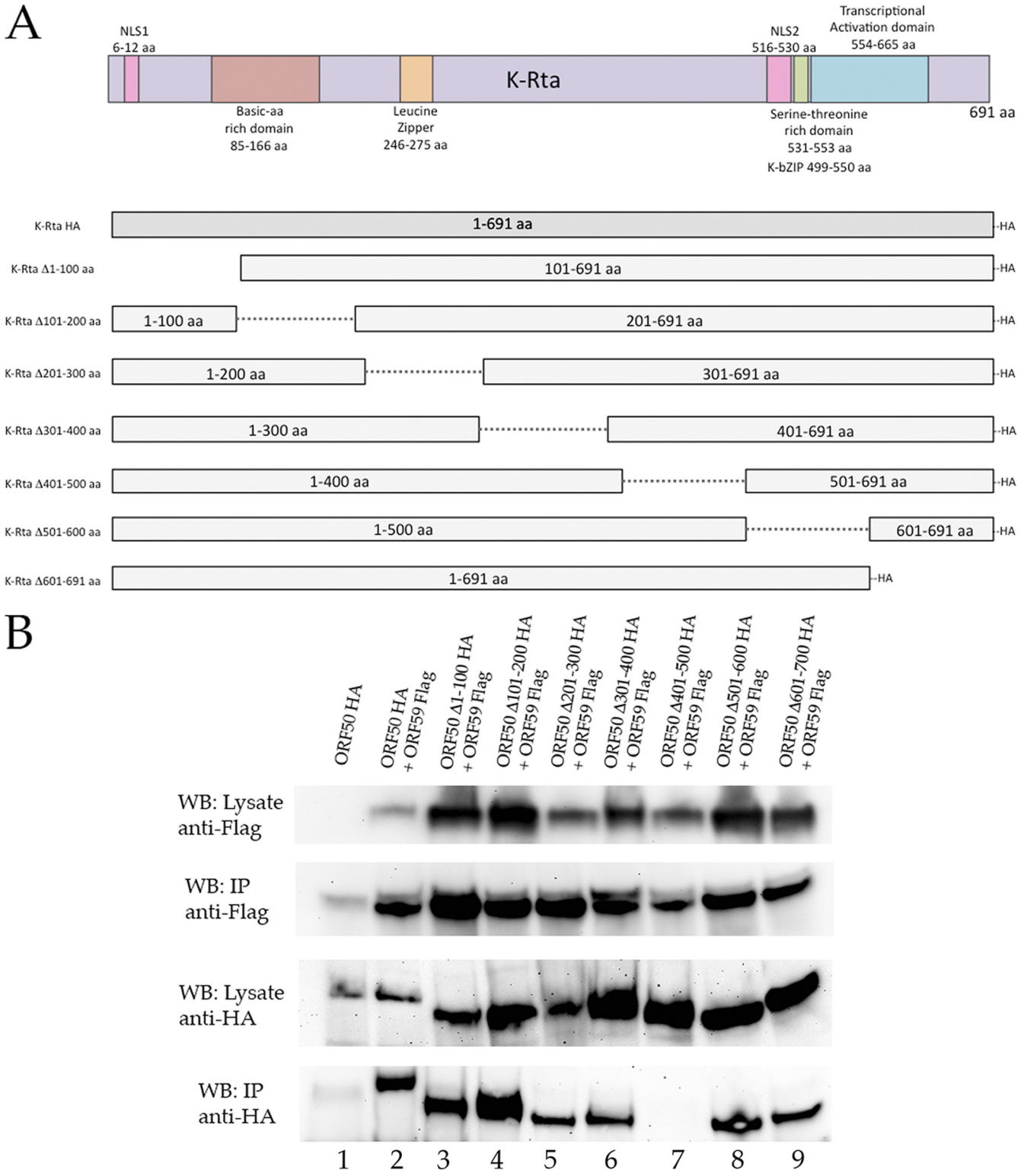

FIG. 5. K-Rta amino acids 401 to 500 are required for interaction with ORF59. (A) Schematic of K-Rta-HA-tagged mutants generated such that 100 amino acids were deleted across the entire ORF. These expression plasmids were individually cotransfected with the full-length ORF59 expression plasmid, and protein complexes were immunoprecipitated using anti-HA specific antibody. (B) Western blots of lysates and immunoprecipitated proteins.

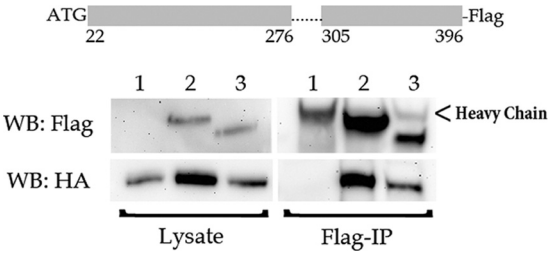

FIG. 6. Dimerization of ORF59 is not required for interaction with K-Rta. A plasmid expressing an ORF59 dimerization mutant was cotransfected into HEK293 cells with a K-Rta expression plasmid. Protein lysates were prepared and incubated with anti-FLAG antibody. Immunoprecipitated proteins were detected using anti-HA antibody after Western blotting. Lanes: 1, K-Rta alone; 2, K-Rta-FLAG plus ORF59-FLAG; 3, K-Rta-FLAG plus ORF59-FLAG dimerization mutant.

expression or function. We cotransfected a K-Rta expression plasmid along with a luciferase reporter plasmid that contained the PAN promoter into Vero cells. To this mixture we added the K-Rta 350-550 expression plasmid (Fig. 7C). The presence of the K-Rta 350-550 expression plasmid had no effect on the ability of K-Rta to activate the PAN promoter (Fig. 7C).

DISCUSSION

For herpesviruses, the initiation of lytic DNA synthesis is emerging as a complex and highly regulated event. Although the core *trans*-acting factors required for lytic DNA synthesis appear to be common across all of the herpesvirus systems studied, there is much variation with respect to regulation and initiation of lytic DNA replication. The factors initiating lytic DNA replication have been elucidated mostly by using the cotransfection replication assay. One of the first initiation fac-

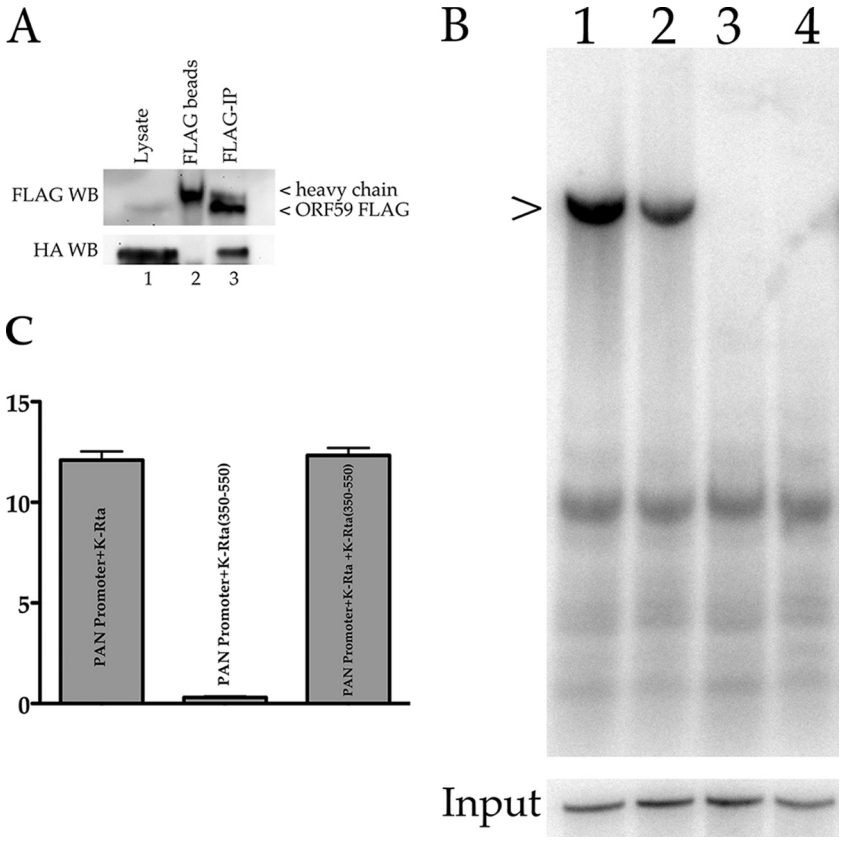

FIG. 7. Dominant negative suppression of complementation of oriLyt-dependent lytic DNA replication by overexpression of the K-Rta interaction domain. (A) Amino acids 350 to 550 of the K-Rta ORF mediate ORF59 binding. An expression plasmid that expressed the HA-tagged aa 350 to 550 peptide of the K-Rta ORF was used in a cotransfection/coimmunoprecipitation assay with full-length FLAG tagged ORF59. Lanes: 1, protein lysate from cotransfection of pK-Rta 350-550aa-HA and ORF59-FLAG; 2, FLAG beads; 3, coimmunoprecipitation from protein lysates prepared from the cotransfection of pK-Rta 350-550aa-HA and ORF59-FLAG. (B) Expression of pK-Rta 350-550aa in the cotransfection replication assay inhibits the amplification of oriLyt. Southern blots of total cellular DNA from Vero cells cotransfected with plasmids expressing all of the required replication proteins, a K-Rta expression plasmid, and cloned oriLyt with or without pK-Rta 350-550aa added to the transfection mixture are shown. Lanes: 1, all replication proteins plus a K-Rta expression plasmid and cloned oriLyt; 2, all required replication proteins, K-Rta expression plasmids, and cloned oriLyt plus pK-Rta 350-550aa (1 μg); 3, all required replication proteins, K-Rta expression plasmids, and cloned oriLyt plus pK-Rta 350-550aa (3 μg); 4, all replication proteins, K-Rta expression plasmids, and cloned oriLyt minus the ORF59 expression plasmid. (C) K-Rta 350-500aa does not affect the transactivation activity of K-Rta. Vero cells were cotransfected with a K-Rta expression plasmid plus a PAN promoter luciferase reporter plasmid with and without the plasmid expression K-Rta 350-500aa. A control transfection containing the PAN promoter luciferase reporter plasmid plus K-Rta 350-500aa was also performed.

tors elucidated was herpes simplex virus type 1 (HSV-1) UL9 (19, 28). UL9 is a protein that possesses helicase activity and interacts with other replication proteins, including ssDNA binding protein and the DNA processivity factor (17, 18, 24). For HCMV, UL84 is a multifunctional protein that interacts with oriLyt and apparently recruits UL44, the DNA processivity factor, to oriLyt via C/EBPα binding motifs in addition to interacting with RNA stem-loop structures within oriLyt (6) (10). Zta, the major transactivator protein for EBV, is capable of reactivating latent virus and controls regulation of EBV lytic replication (13, 21).

Although there appears to be some degree of variation among initiation factors (origin binding proteins), the *cis*-acting oriLyt regions do show functional similarities between the herpesviruses. For example, there is a common requirement for transcriptional activation within lytic origins. HCMV, EBV, and KSHV oriLyt regions contain strong promoters that are activated by major transactivator proteins (1, 9, 14, 20, 27, 31). Additionally, transcription factor binding sites located within oriLyt serve as substrates for viral replication proteins and transactivators and are essential for efficient oriLyt amplification. Hence, there is an emerging hypothesis for a dual role for origin binding proteins as transcriptional activators and potential recruitment factors. In the case of KSHV, the RRE within oriLyt is part of a promoter that subsequently activates K-Rta-mediated transcription. The C/EBPα binding motifs serve as a substrate for K-Rta that may lead to a conformational change in oriLyt and may function in concert with the activation of transcription.

Our studies mapping the interaction domains indicated that the region of ORF59 binding with K-Rta is located within an area of the protein that contains multiple motifs associated with ORF59 dimerization, polymerase interaction, and nuclear localization. Further mutagenesis is required to specifically identify the binding motif. One possibility is that the ORF59 polymerase binding domain is also the K-Rta binding motif. Data suggest that dimerization of ORF59 is not required for an interaction with K-Rta. This result may indicate a function of ORF59 in initiation of DNA synthesis, possibly as a recruitment factor, that is distinct from its role in synthesizing DNA as the defined processivity factor when bound to DNA polymerase. With respect to the K-Rta interaction domain, the region between aa 401 and 500 appears to be necessary for the interaction with ORF59. Expression of the K-Rta interaction domain was able to act as a dominant negative suppressor of complementation of oriLyt-dependent DNA replication, suggesting that the interaction of K-Rta with ORF59 is essential.

Interestingly, a similar interaction between EBV processivity factor BMRF1 and BZLF1 (Zta) was also demonstrated to play a significant role in lytic DNA replication (30). In EBV, the processivity factor was shown to have transactivation activity; however, in contrast to the findings from our studies, BMRF1 appears to decrease the affinity of Zta for its target sequences in oriLyt (29, 30). Nevertheless, these previous studies along with the data presented here identify a novel role for herpesvirus DNA polymerase processivity factors.

We now show that for KSHV, K-Rta is a strong candidate for the lytic DNA initiator protein. The observations that K-Rta interacts with ORF59 and that this interaction is required for amplification of oriLyt are consistent with interactions previously characterized for other herpesviruses. ORF59 expression is regulated by K-Rta via an RRE and an interaction with RBJ-Jκ (16). The processivity factor was first described as a protein that phosphorylates and interacts with DNA polymerase (3). The role of herpesvirus processivity factors is expanding, however, due to the recent findings of their interaction with herpesvirus origin binding proteins in both HSV-1 and HCMV (8, 23–25). This expanding role for processivity factors suggests a novel role in initiation and regulation of lytic DNA replication. For KSHV, although K-Rta has the ability to interact with C/EBPα binding motifs within oriLyt independent of other viral factors, ORF59 may enhance this binding. The interaction of K-Rta with ORF59 may also facilitate the unwinding of DNA at oriLyt and lead to the formation of the entire replication complex at distinct loci within oriLyt.

Initially the protein product from K8, K-bZIP, was a strong candidate for the initiator protein for KSHV due to its homology to EBV Zta (15). However, regulation of KSHV lytic DNA replication is quite complex and involves many interacting factors, including LANA, the latency-associated nuclear protein (22). The interaction between LANA, K-Rta, and K-bZIP and the subsequent interaction of these factors with oriLyt suggest a high degree of modulation of and control of lytic DNA replication for KSHV. It is clear that K-bZIP acts in concert with K-Rta and LANA to initiate lytic DNA synthesis, and our data presented here are the first step in deciphering the balance between these three proteins in the KSHV life cycle.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant CA11521179.

REFERENCES

1. **AuCoin, D. P., et al.** 2004. Amplification of the Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 lytic origin of DNA replication is dependent upon a cis-acting AT-rich region and an ORF50 response element and the trans-acting factors ORF50 (K-Rta) and K8 (K-bZIP). Virology **318:** 542–555.
2. **Chan, S. R., C. Bloomer, and B. Chandran.** 1998. Identification and characterization of human herpesvirus-8 lytic cycle-associated ORF 59 protein and the encoding cDNA by monoclonal antibody. Virology **240:**118–126.
3. **Chan, S. R., and B. Chandran.** 2000. Characterization of human herpesvirus 8 ORF59 protein (PF-8) and mapping of the processivity and viral DNA polymerase-interacting domains. J. Virol. **74:**10920–10929.
4. **Chen, X., K. Lin, and R. P. Ricciardi.** 2004. Human Kaposi's sarcoma herpesvirus processivity factor-8 functions as a dimer in DNA synthesis. J. Biol. Chem. **279:**28375–28386.
5. **Chen, Y., M. Ciustea, and R. P. Ricciardi.** 2005. Processivity factor of KSHV contains a nuclear localization signal and binding domains for transporting viral DNA polymerase into the nucleus. Virology **340:**183–191.
6. **Colletti, K. S., K. E. Smallenburg, Y. Xu, and G. S. Pari.** 2007. Human cytomegalovirus UL84 interacts with an RNA stem-loop sequence found within the RNA/DNA hybrid region of oriLyt. J. Virol. **81:**7077–7085.
7. **Colletti, K. S., Y. Xu, S. A. Cei, M. Tarrant, and G. S. Pari.** 2004. Human cytomegalovirus UL84 oligomerization and heterodimerization domains act as transdominant inhibitors of oriLyt-dependent DNA replication: evidence that IE2-UL84 and UL84-UL84 interactions are required for lytic DNA replication. J. Virol. **78:**9203–9214.
8. **Gao, Y., K. Colletti, and G. S. Pari.** 2008. Identification of human cytomegalovirus UL84 virus- and cell-encoded binding partners by using proteomics analysis. J. Virol. **82:**96–104.
9. **Hammerschmidt, W., and B. Sugden.** 1988. Identification and characterization of oriLyt, a lytic origin of DNA replication of Epstein-Barr virus. Cell **55:**427–433.
10. **Kagele, D., Y. Gao, K. Smallenburg, and G. S. Pari.** 2009. Interaction of HCMV UL84 with C/EBPalpha transcription factor binding sites within oriLyt is essential for lytic DNA replication. Virology **392:**16–23.
11. **Kato-Noah, T., et al.** 2007. Overexpression of the Kaposi's sarcoma-associated herpesvirus transactivator K-Rta can complement a K-bZIP deletion BACmid and yields an enhanced growth phenotype. J. Virol. **81:**13519–13532.

12. **Liao, G., F. Y. Wu, and S. D. Hayward.** 2001. Interaction with the Epstein-Barr virus helicase targets Zta to DNA replication compartments. J. Virol. **75:**8792–8802.
13. **Lieberman, P. M., J. M. Hardwick, J. Sample, G. S. Hayward, and S. D. Hayward.** 1990. The zta transactivator involved in induction of lytic cycle gene expression in Epstein-Barr virus-infected lymphocytes binds to both AP-1 and ZRE sites in target promoter and enhancer regions. J. Virol. **64:**1143–1155.
14. **Lin, C. L., et al.** 2003. Kaposi's sarcoma-associated herpesvirus lytic origin (ori-Lyt)-dependent DNA replication: identification of the ori-Lyt and association of K8 bZip protein with the origin. J. Virol. **77:**5578–5588.
15. **Lin, S. F., D. R. Robinson, G. Miller, and H. J. Kung.** 1999. Kaposi's sarcoma-associated herpesvirus encodes a bZIP protein with homology to BZLF1 of Epstein-Barr virus. J. Virol. **73:**1909–1917.
16. **Liu, Y., et al.** 2008. Kaposi's sarcoma-associated herpesvirus RTA activates the processivity factor ORF59 through interaction with RBP-Jkappa and a cis-acting RTA responsive element. Virology **380:**264–275.
17. **Makhov, A. M., S. S. Lee, I. R. Lehman, and J. D. Griffith.** 2003. Origin-specific unwinding of herpes simplex virus 1 DNA by the viral UL9 and ICP8 proteins: visualization of a specific preunwinding complex. Proc. Natl. Acad. Sci. U. S. A. **100:**898–903.
18. **Marintcheva, B., and S. K. Weller.** 2003. Helicase motif Ia is involved in single-strand DNA-binding and helicase activities of the herpes simplex virus type 1 origin-binding protein, UL9. J. Virol. **77:**2477–2488.
19. **Olivo, P. D., N. J. Nelson, and M. D. Challberg.** 1988. Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. Proc. Natl. Acad. Sci. U. S. A. **85:**5414–5418.
20. **Pari, G. S.** 2008. Nuts and bolts of human cytomegalovirus lytic DNA replication. Curr. Top. Microbiol. Immunol. **325:**153–166.
21. **Petosa, C., et al.** 2006. Structural basis of lytic cycle activation by the Epstein-Barr virus ZEBRA protein. Mol. Cell **21:**565–572.
22. **Rossetto, C., I. Yamboliev, and G. S. Pari.** 2009. Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 K-bZIP modulates latency-associated nuclear protein-mediated suppression of lytic origin-dependent DNA synthesis. J. Virol. **83:**8492–8501.
23. **Strang, B. L., E. Sinigalia, L. A. Silva, D. M. Coen, and A. Loregian.** 2009. Analysis of the association of the human cytomegalovirus DNA polymerase subunit UL44 with the viral DNA replication factor UL84. J. Virol. **83:**7581–7589.
24. **Trego, K. S., and D. S. Parris.** 2003. Functional interaction between the herpes simplex virus type 1 polymerase processivity factor and origin-binding proteins: enhancement of UL9 helicase activity. J. Virol. **77:**12646–12659.
25. **Trego, K. S., Y. Zhu, and D. S. Parris.** 2005. The herpes simplex virus type 1 DNA polymerase processivity factor, UL42, does not alter the catalytic activity of the UL9 origin-binding protein but facilitates its loading onto DNA. Nucleic Acids Res. **33:**536–545.
26. **Wang, Y., H. Li, Q. Tang, G. G. Maul, and Y. Yuan.** 2008. Kaposi's sarcoma-associated herpesvirus ori-Lyt-dependent DNA replication: involvement of host cellular factors. J. Virol. **82:**2867–2882.
27. **Wang, Y., Q. Tang, G. G. Maul, and Y. Yuan.** 2006. Kaposi's sarcoma-associated herpesvirus ori-Lyt-dependent DNA replication: dual role of replication and transcription activator. J. Virol. **80:**12171–12186.
28. **Wu, C. A., N. J. Nelson, D. J. McGeoch, and M. D. Challberg.** 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J. Virol. **62:**435–443.
29. **Zhang, Q., E. Holley-Guthrie, J. Q. Ge, D. Dorsky, and S. Kenney.** 1997. The Epstein-Barr virus (EBV) DNA polymerase accessory protein, BMRF1, activates the essential downstream component of the EBV oriLyt. Virology **230:**22–34.
30. **Zhang, Q., et al.** 1996. Functional and physical interactions between the Epstein-Barr virus (EBV) proteins BZLF1 and BMRF1: effects on EBV transcription and lytic replication. J. Virol. **70:**5131–5142.
31. **Zhu, Y., L. Huang, and D. G. Anders.** 1998. Human cytomegalovirus oriLyt sequence requirements. J. Virol. **72:**4989–4996.